VOLUME XVII October 1961 NUMBER 10

Clinical Proceedings

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*Adapted from Crawford, L. V., and Grogan, F. T.[3]

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references: (1) Clinical Research Division, Mead Johnson & Company. (2) Wahner, H. W., and Peters, G. A.: Proc. Staff Meet. Mayo Clin. *35*:161-169 (March 30) 1960. (3) Crawford, L. V., and Grogan, F. T.: J. Tennessee M. A. *53*:307-310 (July) 1960.

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Connective Tissue Chemistry and the Collagen Diseases

JOHN C. HOUCK, PH.D.*

This writer proposes to describe the chemical anatomy of the connective tissue, indicate the chemistry of the various components of this tissue and then describe the changes in these components which occur as the connective tissue reacts to various stimuli. For all this there are adequate experimental data. The writer will then offer his opinion of the relationship between the connective tissue and collagen, and the various clinical entities referred to as "collagen or connective tissue diseases." These statements, while consistent with pre-existing experimental results, are not demonstrable by them at this time.

THE CHEMISTRY OF THE CONNECTIVE TISSUE

The connective tissue is, like Gaul, divisible into three parts: a) fibers, b) cells, and c) matrix material (table 1). The chemistry of these materials is discussed below.

Fibers

There are three major fibrous proteins found in the connective tissue: elastin, reticulin and collagen. *Elastin* is a wholly insoluble, highly branched fiber consisting of polysaccharides containing large amounts of hexosamine and uronic acid in intimate association with an insoluble protein. The latter material does *not* contain the amino acid hydroxylysine and contains less than 3 per cent hydroxyproline. These fibers, which are elastic in nature, as the name indicates, have no characteristic pattern when examined under the electron microscope, and the unextended fiber demonstrates no evidence of orientation in its x-ray diffraction pattern.

Reticulin is also a branched fiber but that is as far as its similarity to elastin goes. This protein *does* contain hydroxylysine, as well as about

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TABLE 1

The Components of the Connective Tissue

1. Fibers
   a) Elastin, a branched complex protein
   b) Reticulin, a branched precursor of collagen
   c) Collagen, a rodlike protein
2. Cells
   a) Fibroblasts, synthesizing collagen and mucopolysaccharides
   b) Mast cells, synthesizing heparin and histamine
3. Ground Substance
   a) Water, fat, salt, amino acids, sugar, etc.
   b) Acid mucopolysaccharides (hexosamines, uronic acid containing polysaccharides)
   c) Glycoproteins (hexosamine or sialic acid containing proteins)
   d) Mucopolysaccharides (protein-mucopolysaccharides complex)

13 per cent hydroxyproline and 24 per cent glycine. Reticulin is considered by some to be the direct precursor of collagen. The relation of collagen to reticulin may be compared by analogy with that of rope made up of intertwined fibers (collagen) to linoleum in which the fibers (reticulin) are embedded in asphalt (the matrix material of the connective tissues).

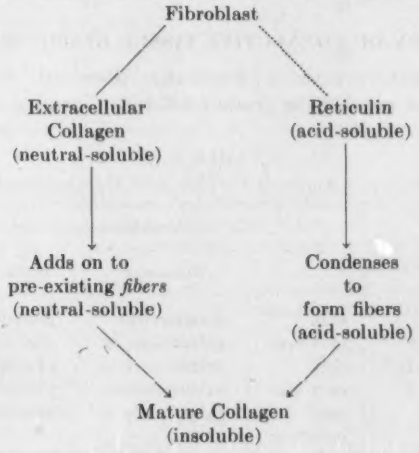
Collagen is a fibrous protein composed of the same amino acids as reticlin in the same concentration. This protein is uniquely rich in hydroxyproline and the amount of this amino acid in various connective tissues is used to determine the amount of collagen. Collagen is found in linear bundles ranging from the fine microfibers of the vitreous humor to the gross "wavy" bundles of the tendon. Collagen has a characteristicly organized fine structure resulting in precise x-ray diffraction patterns, anisotropy and periodic banding about every 640 Angstrom (A) in the electron microscope. This protein, like reticulin, melts to form gelatin, a less structured protein. Collagen fibers are made up of intertwined fibrils, which in turn are composed of protofibrils which are linear polymers of the basic repeating protein unit—tropocollagen. Tropocollagen is a triply stranded protein with a molecular weight of about 360,000. In dilute acids, particularly citrate buffer (pH 3.6), some of this tropocollagen polymer dissolves; the soluble material is called "procollagen" and neutralization of procollagen solutions under proper conditions precipitates fibers which are morphologically and tinctorially indistinguishable from the starting collagen fibers. However, a small amount of this acid-extracted material remains soluble at high ionic strength and neutral pH. Warming or reduction in solution ion concentration will also result in the formation of characteristic collagen fibers from this neutral solution. This type of collagen is similar to that extracted by 0.5M NaCl, or neutral-soluble col-

lagen. The bulk of the collagen in skin, for example, remains insoluble as fibrous collagen (87 per cent of the total). These forms of collagen differ only in their physico-chemical properties and not in their chemical analysis or in the structure of the tropocollagen monomer.

Isotopically labeled proline, when introduced into the diet of rats, becomes incorporated into collagen either as proline or as hydroxyproline; similarly, lysine is incorporated into collagen as either lysine or hydroxylysine. Neither hydroxyproline nor hydroxylysine are incorporated into dermal collagen directly. The isotopic material is found to appear first in the neutral-soluble (0.5M NaCl) collagen, then in the acid-soluble (citrate buffer) collagen and finally in the insoluble collagen. As the concentration of isotope increases in the acid-soluble fraction, it decreases in the neutral-soluble fraction. Eventually, the labeled amino acid is found almost entirely in the insoluble collagen fibers (table 2). This pattern of isotope incorporation, and the mild nature of the solubilizing agents employed, suggests that the mechanism of collagen synthesis is an increasingly profound intermolecular hydrogen binding between tropocollagen molecules. This increasing internal binding decreases solubility so that with time, neutral-soluble collagen molecules become soluble only in dilute acid and then finally become insoluble. From a clinical point of view, it should be noted that the tensile strength of healing wounds is directly and linearly related to the dermal concentration of insoluble

TABLE 2

Possible Biosynthetic Pathways of Collagen



collagen only. This is the form of the protein polymer which provides structural support to the tissues.

Cells

Two major cell types, exclusive of blood elements, have been linked to the specific biochemical properties of the connective tissue: fibroblasts, which synthesize collagen and acid mucopolysaccharides, and mast cells which contain heparin, histamine, and 5-hydroxytryptamine. Certain fibroblasts may have specialized functions, such as chondroblasts and osteoblasts; inclusion of polyanionic materials into the fibroblast results in a cell type closely resembling the mast cell.

Matrix Material

Matrix material consists of water and electrolytes moving through the ground substance. This latter material contains proteins similar to serum albumin, glycoproteins and eight different polysaccharides containing hexosamine. These polysaccharides are often grouped under the term "mucopolysaccharide" (table 3). One of these, hyaluronic acid, is found free in the ground substance of the skin, while another acid mucopolysaccharide, derman sulfate (chondroitin sulfate B), is bound to collagen. Heparin is largely confined to the interior of the mast cell. A monosulfated heparin is excreted in quantity by patients with Hurler's syndrome. Another acid mucopolysaccharide, keratosulfate, which is found primarily in cartilage, increases in concentration with age in the human but is found in extremely large quantities in the cartilage from patients with Marfan's syndrome.

THE CHEMISTRY OF CONNECTIVE TISSUE RESPONSE TO STIMULI

When the connective tissue is chemically "dissected" by sequential extraction to remove most of the ground substance, neutral-soluble collagen,

TABLE 3

Composition and Source of Various Acid-Mucopolysaccharides

Name	Tissue	Acid-Mucopolysaccharides Composition			
		Hexosamine	Uronic Acid	SO_4	Acetyl
hyaluronate	skin	glucosamine	glucuronic	0	+
chondroitin sulfate A	cartilage	galactosamine	glucuronic	1	+
chondroitin sulfate B	skin	galactosamine	iduronic	1	+
chondroitin sulfate C	cartilage	galactosamine	glucuronic	1	+
chondroitin	eye	galactosamine	glucuronic	0	+
keratosulfate	cartilage			1	
heparin	lung	glucosamine	glucuronic	3	0

and the acid-soluble collagen or procollagen separately, the residue from this extraction process is primarily insoluble collagen. The total amount of collagen in each fraction may be calculated from the hydroxyproline concentration; the collagenous nitrogen may be calculated from the fact that collagen contains more nitrogen than any other protein (18.6 per cent). The noncollagenous protein may be calculated from the difference between the total nitrogen and the collagenous nitrogen. Animal experiments have shown that there is a decrease in soluble collagen with *age*, whereas the amount of soluble noncollagenous protein goes through maximal value at a body weight equivalent to a young adult. It has also been demonstrated that the isotonic saline-soluble dermal hexosamine (contributed by mucopolysaccharides and glycoproteins), used as a measure of ground substance, decreases precipitously after the animal reaches adulthood. With further aging, an increasing proportion of the hexosamine becomes insoluble. During maturation, as distinct from aging, all the hexosamine remains soluble. Thus a distinction between maturation and aging may be indicated by these data with respect to insoluble hexosamine, collagen and noncollagenous protein (table 4).

TABLE 4

The Effect of Age upon the Percent of the Total Insoluble Dermal Hexosamine, Collagen and Noncollagenous Protein

Rat Weight (Gm.)		Insoluble Hexosamine	Insoluble Collagen	Insoluble Protein
100	Maturation	0	65	50
150		0	71	35
200		0	82	22
270	Aging	35	85	2
350		44	87	21
500		50	95	46

TABLE 5

The Distribution of Dermal Collagen (mg./Gm.) in Skin Distant from the Site of Injury Seven Days after Injury

	Extract:		
	Neutral-Soluble	Acid-Soluble	Insoluble
1) Normal Control	19	8	204
2) Croton oil inflammation	9	30	140
3) NaOH inflammation	10	31	150
4) Sham adrenalectomy	10	28	138
5) Cortisol administration (3 mg./Kg.)	9	33	153

TABLE 6

The Total Dermal Collagen and Hexosamine Concentration (mg/Gm) of Necrotic Lesions Produced by Various Amounts of Croton Oil

Days after Injury	100% Oil		75% Oil		50% Oil		25% Oil	
	Hex.*	Collagen	Hex.*	Collagen	Hex.*	Collagen	Hex.*	Collagen
0	1.8	225	1.8	220	1.8	225	1.8	225
3	2.9	160	2.8	150	4.5	67	3.6	90
7	4.2	140	4.2	110	7.0	59	5.0	47
14	4.9	155	3.7	120	6.3	72	9.4	63
24	1.8	205	2.0	220	1.9	139	2.2	150

* Hex. = hexosamine (mg./Gm. skin).

TABLE 7

The Dermal Collagen and Hexosamine Content of Necrotic Lesions With and Without Topical Application of Trypsin

Days after Injury	Collagen (mg./Gm.)		Hexosamine (mg./Gm.)	
	Control	Trypsin	Control	Trypsin
0	220	220	1.8	1.8
3	62	166	4.6	2.8
7	59	168	7.1	2.5
14	72	155	6.4	2.3
23	96	168	2.1	1.9

The hydroxyproline or collagen content of rat skin distant from the site of local inflammation produced by intradermal injections of *sodium hydroxide* has been shown to be profoundly decreased. This decrease in dermal collagen occurring in response to distant local inflammation has been shown to be specific for this protein and not due to a general catabolic response to the stress of injury; it involves replacement of insoluble collagen by other insoluble proteins and is not demonstrable histochemically. When local injury is produced by *croton oil*, similar changes in the collagen content of the uninjured skin distant from the site of local injury are found. The uninjured skin from animals injured with either irritant shows a decrease in neutral-soluble collagen and insoluble collagen and an increase in acid-soluble collagen without apparent alterations in the distribution of hexosamine or nitrogen.

Cortisol administration decreases both the neutral-soluble and insoluble collagens, while increasing the acid-soluble material. This hormone, in small amounts, does not affect either the hexosamine or the nitrogen content

of the skin. The distant dermal collagen response to local inflammation described above may be quantitatively duplicated by cortisol administration, and therefore probably is a reaction, specific for collagen, to the trauma (and stress?) of local injury (table 5).

Local to the site of injury, there is a profound increase in the tissue concentration of hexosamine, and a marked decrease in collagen with only the most minor change in dermal nitrogen. If proteolytic enzymes are applied topically to hydrolyse the fibrin micro-clots which have formed in both the capillaries and the lymphatic channels, the circulating concentration of hexosamine and hydroxyproline is increased, while the tissue content of these materials is decreased. This type of evidence indicates that with local inflammation and necrosis the products of the breakdown of the connective tissue drain into the circulation.

The effect of irritant concentration discloses two types of response of the host to the local damage produced by chemically induced inflammation: 1) when the wound is too profound to be rapidly isolated and sloughed, the circulation into the wound is partially restored to effect the dilution of both the irritant and the products of necrosis, to drain these products into the circulation, and to permit the "healing elements of the blood" to do what they can; and 2) when the lesion is sufficiently small to be rapidly isolated and sloughed. Table 6 shows that with increasing irritant concentration, the percentage of collagen lost and hexosamine gained during inflammation is decreased. Similarly, with increasing irritant concentration, more hydroxyproline is found in the circulation. Finally, if a lesion such as that produced by 50 per cent croton oil (which is rapidly isolated by fibrin blockade), is treated topically with trypsin, the biological continuity of the tissue is partially restored via proteolysis of the fibrin blocking the lymphatic channels and the capillaries, and the wound so treated loses less collagen and gains less hexosamine than the untreated lesion, and hydroxyproline is found in the circulation (table 7).

Preliminary evidence suggests that the circulating concentration of bound hydroxyproline is elevated in the human child or adult by osteomyelitis, osteogenesis imperfecta, arthritis, and collagen degeneration in general. The possible clinical importance of the intrusion of connective tissue elements into the circulation is described below.

Sodium dilantin administration to rats results in an increase in insoluble collagen (50 per cent) and in acid-soluble collagen while showing a decrease in neutral-soluble collagen. This type of response to dilantin involves the increase in connective tissue collagen and keratin, and considerable epithelization.

These responses of the connective tissue to age, dilantin administration, inflammation, and cortisol all indicate that a considerable redistribu-

tion of the various chemical components of this tissue occurs under physiological stimulus. The soluble and insoluble collagen and noncollagenous proteins as well as the water and fat content of the skin are in constant flux in response to various stimuli, indicating that this tissue is not as metabolically inert as has been thought previously.

Specifically, with maturation there is a decrease in the soluble collagen of the dermis and an increase in total soluble noncollagenous proteins. With aging, both soluble proteins are decreased, and there is a precipitous decrease in ground substance and a sudden appearance of insoluble mucopolysaccharide and glycoprotein. Dilantin, which results in a profound increase in insoluble collagen, also causes a decrease in neutral-soluble collagen and an increase in acid-soluble collagen. Local inflammation results in a specific loss in insoluble collagen from the apparently uninjured skin, while there is a decrease in the neutral-soluble collagen and an increase in acid-soluble collagen. Thus similar changes in the soluble collagens result in increased insoluble collagen on the one hand, and decreased insoluble collagen on the other. This striking inconsistency is worthy of some thought. What is the mechanism of the dermal collagen decrease in the skin distant from the site of injury? What stimulates this distant dermal collagen response, and where do the products of this reaction go?

The specific stimulus for this collagen response may be cortisol which would be released in response to the trauma of inflammation, since the effect of this corticosteroid upon the dermal chemistry is strikingly similar to that of local inflammation. Ganglionic blocking agents decrease this distant dermal collagen response, probably by blocking the dermal nerve-central nervous system-pituitary-ACTH-cortisol pathway appropriate to the general stress response. Regardless of the validity of this explanation for the stimulation of the distant dermal collagen response to local injury, the questions of how it occurs and where the collagen goes remain unanswered.

SPECULATIONS REGARDING THE CHEMISTRY OF THE RESPONSE OF CONNECTIVE TISSUE TO STIMULI

Because of the specificity of the loss of insoluble dermal collagen with injury, this reaction most probably occurs in response to collagenolysis by a dermal proteolytic enzyme specific for collagen. In the face of this loss in insoluble collagen, the fibroblasts might be stimulated to synthesize neutral-soluble collagen. This material would be rapidly transformed by physical-chemical interaction to form acid-soluble collagen. Subsequent to the maximal formation of acid-soluble collagen, insoluble collagen is maximally synthesized, as shown by the dilantin experiments.

The kinetics of these changes which eventually result in the synthesis of collagen suggest that neutral-soluble collagen is rapidly aggregated to form insoluble collagen. If collagenolysis of the insoluble collagen by a dermal collagenase stimulates collagen synthesis by the fibroblasts, then the changes in soluble dermal collagen produced by cortisol administration or chemical injury may proceed from this stimulation. The slowest, or rate-limiting, step of this pathway is the formation of the insoluble fibers. The kinetics of the distant dermal collagen response to local inflammation indicate that the insoluble collagen decreases maximally immediately after injury, as does the neutral-soluble collagen. A few days after the onset of injury the concentration of acid-soluble collagen is maximally increased. The conclusion of this process is indicated by an increase in insoluble collagen followed by an increase in neutral-soluble collagen and finally a decrease in the acid-soluble material. The kinetics of these changes suggest that a steady-state is formed in which collagenolysis is balanced against the accumulation of acid-soluble collagen, which slowly aggregates to form fibers.

In view of the large amounts of proteolytic enzymes found in both dermis and fibroblasts, and the known responsiveness of these cells to adrenal cortical hormones, it is possible that cortisol causes the release of a collagenase from the fibroblasts. Thus this cell would be both degrading and synthesizing collagen simultaneously. Such a concept receives some support from pathology, i.e., the association of chondroblasts with cartilage and bone resorption as well as with synthesis is well known. Destruction of cartilage requires collagenolysis, and appears histologically to take place in close contact with inflammatory connective tissue such as synovial pannus, although in active lesions it is frequently apparent that the chondrocytes are necrotic well in advance of the pannus. The small blood vessels within the active pannus are often obliterated by initial hypertrophy of fibrin thrombosis, suggesting the possibility that the products of ischemic tissue might be concerned in local autolysis. This pathological process is almost always found in rheumatic disease, and may well be started and maintained via the release of a collagenase from the chondroblasts.

The insoluble dermal collagen lost in response to local inflammation must be made soluble in such fashion as to permit the products to drain away from the skin via the lymphatics. The lymph nodes of experimental animals show a threefold increase in hydroxyproline subsequent to the decrease in insoluble dermal collagen with local inflammation. The loss in collagen from uninjured skin distant from the site of local injury is not associated with a measurable increase in circulating hydroxyproline, however. The route for the disposal of this collagen is unknown.

Regardless of the precise route, collagen and/or the products of col-

lagenolysis come in contact with the reticuloendothelial system, while the products of local necrosis drain into the circulation. The release of connective tissue elements into the circulation and the reticuloendothelial system with inflammation suggests the possibility that one of these materials might stimulate antibody formation! Collagen is known to be mildly antigenic, and this structural protein is not a normal constituent of the circulation. The possibility that an animal becomes reactive to his own collagen which has been released via inflammation and produces antibodies which leak into the connective tissue is interesting in light of the suggested clinical correlation between infection and rheumatoid arthritis.

Pathological evidence now indicates that vasculitis is a primary manifestation of rheumatoid arthritis, as it is in the other connective tissue diseases. These vascular lesions demonstrate a particular predilection for venules, which tend to become obliterated by necrosis; this appears to be secondary to microcirculatory insufficiency. The reaction between anticollagen antibody and collagen could take place in the venule wall, with the various pathological changes proceeding from the resulting minute lesion. Presumably collagen fibrogenesis would be inhibited by such an antibody, and the primary areas of collagen replacement are found in the articular tissue so often involved in arthritis. The pathological cycle of necrosis → collagenolysis → antibody → antibody injury and then necrosis again, may describe both the etiology and the mechanism of the "collagen diseases."

The writer has attempted to describe the chemistry of connective tissue and its components, and the effect of various physiologic, pharmacologic and traumatic stimuli upon the chemical anatomy of this tissue.

The speculations relating the observed experimental data to the collagen diseases are difficult to prove, but at the present time are at least consistent with most of the available data. It is hoped that the factual portion of this manuscript will be informative, and that the speculations will be at least provocative, even if not correct.

Recent Advances in Hemoglobin Electrophoresis

LIEUTENANT COMMANDER HOWARD A. PEARSON, MC, U. S. N.*

Although sickle-cell anemia and sickle-cell trait were delineated as clinical entities many years ago, the modern era of investigation of these conditions began in 1949. At that time, Linus Pauling and his associates[1] demonstrated an abnormal hemoglobin in sickle-cell anemia and advanced the concept of "molecular disease." They utilized the technique of electrophoresis in their studies, using as the basis for study the well known property of protein molecules in solutions of varying pH to have definite electrical charges, depending upon their amino-acid composition. If these molecules are placed in an electrical field, their migration or mobility is dependent upon the molecular charge. This is the basic principle of electrophoretic-analytic techniques.

Pauling and his associates used the complicated moving boundary or Tiseleus technique of electrophoresis. The development of the simple and inexpensive filter paper electrophoretic apparatus provided the clinician with an extremely valuable and practical investigative tool. After sickle hemoglobin (Hemoglobin S), new hemoglobin species were rapidly discovered, such as Hemoglobins C, D, E, H, I and J. The alphabet has been nearly exhausted, since more than two dozen different species of human hemoglobins have been described. Figure 1 shows the patterns of some of the better categorized variants. Fortunately, from a practical point of view, only a few of these are of clinical significance. The rest are of great anthropologic and genetic interest but are rarely seen even in a busy hematologic practice.

New and exciting work has been recently done on the basic structure of the hemoglobin molecule. This molecule is spherical and is made up of a complex protein called globin, and four iron-porphyrin heme groups. In all hemoglobin variants, the changes occur in the protein or globin moeity; the heme portion remains unaltered.

Globin consists of two types of long polypeptic chains which are designated α and β. The chemical "formula" of hemoglobin can, therefore, be designated as $\alpha_2\beta_2$. Most of the clinically important hemoglobins have abnormal β chains only. For example, the "formula" for sickle hemoglobin could be designated $\alpha_2\beta_2^s$ and that for hemoglobin C would by $\alpha_2\beta_2^c$.

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This molecular "detective work" has been pursued to even finer detail by Dr. Vernon M. Ingram.[2] Dr. Ingram, by means of precisely controlled trypsin digestion, has split the complex hemoglobin molecule into 28 peptide fragments, each containing 8 to 10 amino acids. These peptide fragments were analyzed by two dimensional chromatography and electro-

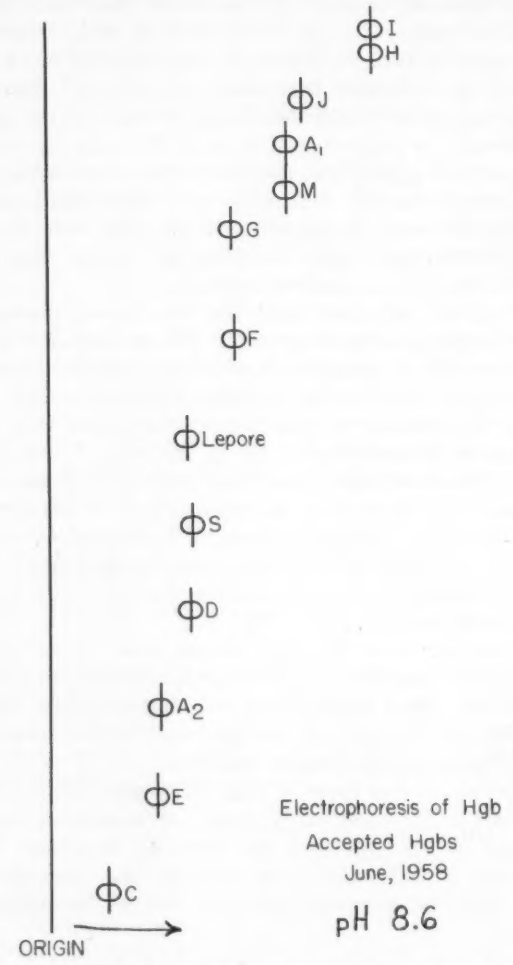

FIG. 1. Electrophoresis of hemoglobin, pH 8.6. The relative electrophoretic mobilities of some of the better defined varieties are depicted.

phoresis, producing a pattern which Ingram called the "fingerprint" of hemoglobin. He found that Hemoglobins A, S and C differed in only *one* of the 28 peptides. He then performed precise sequential amino-acid analysis of this one different peptide of Hemoglobins A, S and C. Each consisted of eight amino acids. The sequence of this is outlined in figure 2. In position 6 of Hemoglobins S and C, valine or lysine is present instead of the glutamic acid in position 6 of Hemoglobin A. These sub-

Hb A. Valine-Histidine-Leucine-Threonine-Proline-*GLUTAMIC ACID*-Glutamic acid-Lysine . . .

Hb S. Valine-Histidine-Leucine-Threonine-Proline-*VALINE*-Glutamic acid-Lysine . . .

Hb C. Valine-Histidine-Leucine-Threonine-Proline-*LYSINE*-Glutamic acid-Lysine . . .

FIG. 2. Scheme of the amino acid sequence in the number 4 peptide fragment. Note that there are differences only in the number 6 position. (After Ingram).

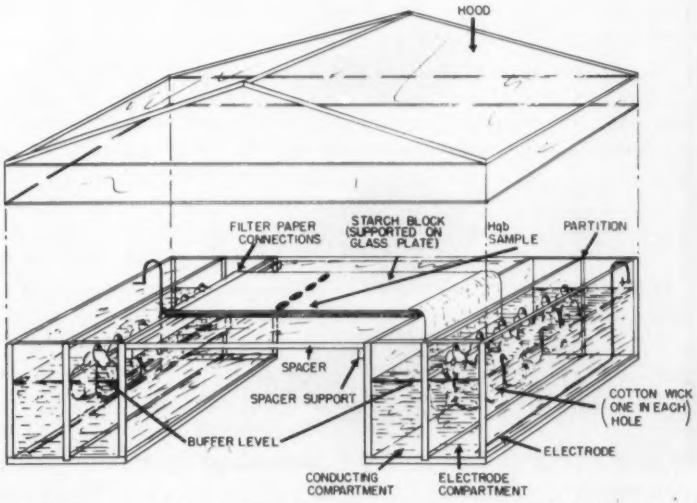

FIG. 3. Starch block electrophoretic apparatus (Courtesy of U. S. Armed Forces Medical Journal).

stitutions cause changes in molecular charge and are fundamentally the basis for the differences in electrophoretic behavior of the hemoglobins. The fact that the same precise molecular locus is affected in these hemoglobins lends striking confirmation to the clinical impression that the genes responsible for them are allelic. This discovery provides real insight into the mechanism of gene action.

Although standard filter paper electrophoresis is a useful analytic tool, it has several inherent disadvantages. First is the fact that several hemoglobin variants have identical electrophoretic mobility; for example, Hemoglobins S and D or Hemoglobins F and G. To differentiate these, other techniques must be utilized. For example, S can be differentiated from D by means of the sickling phenomenon, solubility studies, or agar electrophoresis.[3] F can be differentiated from G by means of the alkali denaturation process,[4] and again by agar electrophoresis.

An even more serious criticism of filter paper electrophoresis is its lack of sensitivity. Hemoglobin components constituting less than 15 to 20 per cent of a mixture cannot be regularly detected. To overcome this handicap other supporting media were developed for hemoglobin electrophoresis. Considerable recent work has been done utilizing Kunkel

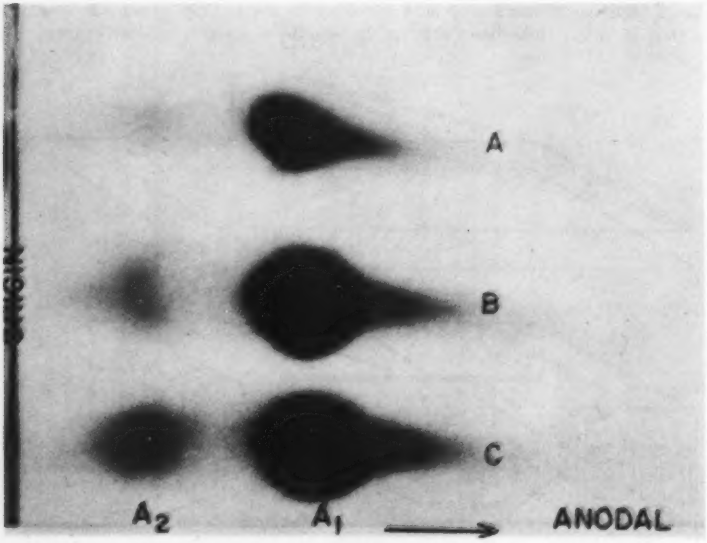

FIG. 4. Starch block electrophoresis of hemoglobins pH 8.6. a) Normal: Hgb. A_2 = 2.4 per cent. b) Thalassemia trait: Hgb. A_2 = 4.3 per cent. c) Thalassemia trait: Hgb. A_2 = 5.7 per cent. (Courtesy of U. S. Armed Forces Medical Journal).

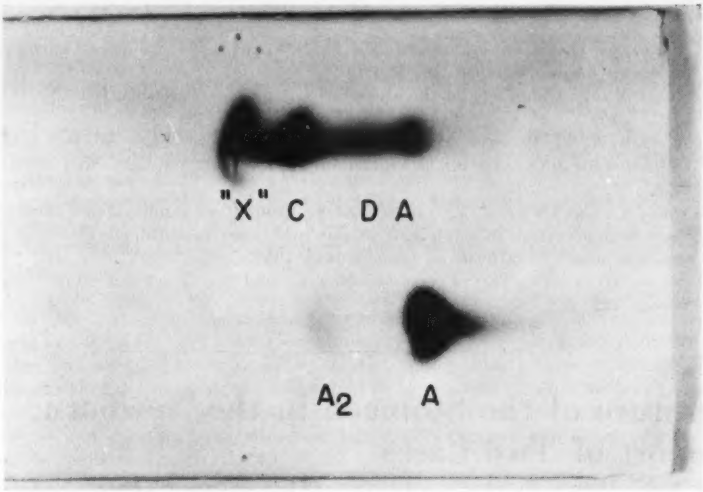

FIG. 5. Starch block electrophoresis of patient with four major hemoglobin components.

and Wallenius' technique of starch block electrophoresis.[5] Figure 3 depicts the apparatus used. A large block of sedimented starch is used as the supporting medium. Since starch is a nearly inert medium, and since the block can be loaded with relatively large amounts of hemolysate, very minute components can be detected.

When hemoglobin from a normal person is subject to starch block electrophoresis, it is found that instead of being homogeneous, there are two definite components (fig. 4). The slow minor component is called Hemoglobin A_2. In normal persons, it acoounts for 1 to 3 per cent of the total. However, in most patients with thalassemia trait, A_2 is elevated to 4 to 8 per cent, providing for the first time a reliable and relatively specific test for this condition.

Figure 5 is another illustration of the utility of starch block electrophoresis. A Negro patient was found to have a moderately severe hemolytic anemia. Filter paper electrophoresis demonstrated a C-A pattern. However, on starch block, a very complex and intriguing pattern consisting of four different components was observed. Hemoglobin "X" on this pattern is a new previously undescribed species.

Using this type of sensitive test, more and more is being learned about the inheritance of human hemoglobin. It is indeed no exaggeration to say that the study of hemoglobin is bringing us close to an understanding of human genetics, heretofore possible only in fruit flies and peas.

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Rupture of the Stomach in the Newborn: Report of Two Cases

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Spontaneous rupture of the stomach in the newborn is a rare entity. According to Vargas and associates,[1] the first such case was reported in 1825 by Siehold, and through 1938 a total of only 9 cases had been reported; the etiology of seven was not elucidated, but two occurred in premature infants with perforated ulcers. In 1946 a case was thoroughly investigated by Pendergrass and Booth[2] in which neither an ulcer nor a congenital defect was present. Another case, studied in 1951 by Kellogg and his associates,[3] clearly demonstrated a rupture due to peptic ulceration. The first infant to survive a ruptured stomach, the result of a congenital defect, was reported by Rosenberg and Heath[4] in 1946.

The site of rupture was not given much attention until 1954 when a review of the literature and classification according to site was carried out by Northway and others.[5] In addition, Meyer[6] in 1957 showed that the majority of ruptures associated with congenital defects occurred along the greater curvature. Others have also noted the predilection of such ruptures for the greater curvature.[7, 8]

The two cases of ruptured stomach seen in the newborn at Children's Hospital since 1954 illustrate quite different symptomatology.

CASE REPORTS

Case 1.

The patient was a 1 day old white male infant who was born by cesarean section following a transverse arrest; pregnancy and labor were otherwise normal. Eighteen

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hours after birth he began having episodes of melena and hematemesis and was found in shock. He received a blood transfusion and vitamin K, but did not improve and was transferred to Children's Hospital. At the time of admission he was a well developed, pale infant with a temperature of 97.6°F., a pulse of 120 per minute, and respirations of 56 per minute. Dark blood was coming from the nostrils and mouth, but no area of active bleeding was found. Heart and lungs were normal. The liver was palpable 1.5 cm. below the right costal margin and the spleen was just palpable below the left costal margin. Neurologic examination was normal. Laboratory data included hemoglobin concentration of 11.3 Gm. per 100 ml., hematocrit 32 per cent, and leukocyte count 14,100 per cu. mm. with a normal differential; prothrombin time was 77 per cent of normal, a later value being 22 per cent of normal. Serum electrolytes were normal.

Upon admission the patient was given oxygen, 40 mg. of Mephyton®, fresh whole blood, and later fresh plasma. Eight hours after admission abdominal distention, absent bowel sounds, and icterus were noted. An exploratory laparotomy performed at that time revealed a tear along the anterior border of the greater curvature of the stomach. During the repair the spleen had to be removed to allow better exposure. Although the bleeding subsided 28 hours postoperatively, the patient developed cyanosis and grunting respirations, and died with terminal symptoms of cyanosis and Cheyne-Stokes respirations.

At autopsy the gastric mucosa was diffusely purplish gray with indistinguishable rugae. Microscopic sections showed diffuse hemorrhage throughout the submucosa and focally in the muscular layer. Hemorrhage had disrupted the mucosa at several points; there was no indication of reaction to a toxin. The final diagnoses were:

1. Gastric hemorrhage, massive
2. Status postoperative, repair of spontaneous stomach perforation
3. Status postoperative, splenectomy
4. Hemorrhage, submucosa, esophagus and trachea
5. Alveolar hemorrhage

Case 2.

The patient was a 17 day old white male infant who was admitted to Children's Hospital because of cyanosis. At birth and for the following four days the infant had had difficulty in breathing and intermittent cyanosis. However, he improved and was discharged. Again, four days prior to admission, cyanosis was noted, especially during feeding. Two days later it became persistent and a slight cough developed. By the time of admission respiratory difficulty was marked.

Significant physical findings, in addition to cyanosis, included mongoloid facies and stigmata, moderate dehydration, marked use of the accessory muscles of respiration, substernal retraction, and fine moist rales over the entire lung fields. Heart sounds were heard best in the right chest. Examination of the abdomen was within normal limits; there were no palpable masses. Initial impressions were diffuse bronchopneumonia, possible congenital heart defect, mongolism, and dextrocardia. The last was confirmed by x-ray which showed, in addition, opacity in the lower third of the left chest suggestive of consolidation. By the second day the patient's condition seemed improved, but the liver edge was palpable at the level of the umbilicus. The patient was given digitalis. On the third day, after a barium swallow, he became extremely cyanotic and had to be given oxygen in an incubator. Although his condition seemed slightly improved the next day, he was covered with red ecchymotic areas and had petechiae on his face. He vomited coffee ground material and had a loose gray-brown bowel movement. Re-examination at this time showed the abdomen

to be distended and firm, and cyanosis to be present even when in oxygen. He died on the fourth hospital day.

At autopsy there was a 4 by 4 cm. irregular perforation involving the posterior aspect of the greater curvature of the stomach. There was omentum overlying and adhering to the perforation; covering the omentum were flecks of creamy green material. On section the entire gastric wall was congested and showed evidence of subacute inflammation. In the area of the perforation there was massive hemorrhage throughout all layers of the gastric wall. The pylorus was unremarkable except for one small area of acute inflammation, and two other sections showed acute congestion, hemorrhage, and disseminated inflammatory cells. The final diagnoses were:

1. Gastric perforation
2. Peritonitis
3. Bronchopneumonia
4. Hydrothorax
5. Mongolism
6. Hypertrophy of the right ventricle

DISCUSSION

Etiology

The causes or associated conditions accompanying rupture of the stomach in the newborn are varied. Mann and his associates[9] find the following to be most common: 1) peptic ulcer, 2) injury from vigorous gavage, 3) congestion following septicemia, and 4) congenital defects in the musculature of the stomach. Other cases of rupture have been attributed to birth injuries and oxygen therapy.[10] Greene and Gose[7] seek explanation on a more local basis: 1) unusual external pressure, e.g., that exerted in passage through the birth canal, 2) distention of the stomach with or without obstruction, 3) trauma to the mucosa, and 4) congenital defects of the musculature. A hormonal mechanism is also postulated implicating the "hypothalamic-pituitary-adrenal complex" which promotes high corticosteroid levels and contributes to rupture of the stomach.[8] Brain injuries influencing this mechanism via the autonomic nervous system are known to cause acute peptic ulceration in infants.

The site of rupture is related somewhat to the etiology. Potter[11] believes that rupture due to a gavage tube occurs invariably along the greater curvature. Spontaneous rupture accompanying septicemia, reported for the first time by Dunham and Goldstein[12] in 1934 and later by other investigators,[1, 9, 11] is less specifically associated with any particular site. Rupture in which congenital defect of the musculature is found occurs more frequently along the greater curvature.[11, 13] That associated with peptic ulceration occurs more often in the pylorus or along the lesser curvature.

Diagnosis

Signs and symptoms of this condition are not clear-cut. Refusal of food may be an early sign. Vomiting usually occurs, but it may vary from regurgitation of small amounts of bile-stained material to frank hema-

temesis. Signs of peritonitis are usually present; abdominal distention and rigidity, shallow and rapid respirations, reduced bowel sounds, and cyanosis due to splinting of the diaphragm are outstanding. Difficulty in irrigating the stomach may suggest the possibility of leakage through a perforation. Shock due to fluid loss from the interstitial and intravascular compartments rapidly or eventually becomes manifest.

The x-ray may show air free in the peritoneal cavity, under the diaphragm or above the dependent regions. Air-fluid levels may also be evident. Several distended loops of intestine may be seen. Air in the stomach may be conspicuously absent. Whenever pneumoperitoneum is seen in the upright film of an infant with a distended abdomen and dyspnea, rupture of a hollow viscus (especially the stomach) must be considered.

Treatment

Success in the treatment of rupture of the stomach in the newborn depends on early diagnosis, prompt surgery to close the defect, irrigation of the peritoneal cavity, and vigorous antibiotic therapy since the most serious complication is overwhelming peritonitis. Fluid and electrolyte balance must also be maintained carefully. If all goes well, oral feeding may be started in 72 hours after surgery.

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